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Comparative copper sensitivity between life stages of common subantarctic marine invertebrates

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Abstract

The development of environmental guidelines in the Antarctic and subantarctic is essential, because expansion of research, tourism, and fishing is placing these regions at increasing risk of contamination. Data are currently insufficient to create the region-specific guidelines needed for the unique conditions in these areas. To develop the most appropriate environmental guidelines, data from the most sensitive life stages of a species should be included to ensure effective protection throughout its life cycle. It is generally accepted that early life stages are more sensitive to contaminants. We compared the toxicity of copper between juvenile and adult life stages of 4 subantarctic marine invertebrates using sublethal and lethal endpoints. For 2 of the species tested, juveniles were more sensitive than adults. (The 7-d median effect concentration [EC50] values for the gastropod Laevilittorina caliginosa were 79 μg/L at the juvenile stage and 125 μg/L at the adult; for the flatworm Obrimoposthia ohlini, values were 190 μg/L at the juvenile stage and 300 μg/L at the adult.) For the isopod Limnoria stephenseni, juveniles were either more sensitive or of equal sensitivity to adults (7-d EC50 values: juvenile 278 μg/L and adult 320 μg/L). In contrast, for the bivalve Gaimardia trapesina, adults appeared to be more sensitive than young adults (7-d EC50 values: juvenile 23 μg/L and adult < 10-20 μg/L). Although no consistent trend in the sensitivity of life history stages was observed, the present study contributes important information for the development of water quality guidelines in polar regions.

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COMPARATIVE COPPER SENSITIVITY BETWEEN LIFE STAGES OF COMMON SUBANTARCTIC MARINE INVERTEBRATES

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Abstract

The development of environmental guidelines in the Antarctic and subantarctic is essential, as expansion of research, tourism and fishing places these regions at increasing risk of contamination. Due to unique conditions in these areas, region specific guidelines are required, however there are currently insufficient data to do this. To develop the most appropriate environmental guidelines, data from the most sensitive life stages of a species should be included to ensure effective protection throughout its life cycle. It is generally accepted that early life stages are more sensitive to contaminants. In this study, we compared the toxicity of copper between juvenile and adult life stages of four subantarctic marine invertebrates using sublethal and lethal endpoints. For two of the species tested, juveniles were more sensitive than adults (7 d EC50s: gastropod *Laevilittorina caliginosa* juvenile 79 µg/L; adult 125 µg/L; flatworm *Obrimoposthia ohlini* juvenile 190µg/L; adult 300 µg/L). For the isopod, *Limnoria stephenseni*, juveniles were either more sensitive or of equal sensitivity to adults (7 d EC50s: juvenile 278µg/L; adult 320 µg/L). In contrast, adults appeared to be more sensitive than young adults for the bivalve *Gaimardia trapesina* (7 d EC50s: juvenile 23µg/L; adult <10-20 µg/L). While no consistent trend in the sensitivity of life history stages was observed, this study contributes important information for the development of water quality guidelines in polar regions.

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INTRODUCTION

Though largely still considered a pristine environment, many decades of human activity has left the subantarctic and Antarctic regions exposed to and impacted by a range of contaminants. Legacy waste tip and fuel spill sites around subantarctic and Antarctic research stations generally contain elevated concentrations of contaminants including metals [1-3]. Effects of climate change including increased rainfall and sea-level rise, may increase the mobilisation of contaminants from such sites and enhance their movement to intertidal zones [4], while other effects such as increased temperature and decreased pH could alter toxicity [5, 6].

Species and ecosystems at high latitudes may be more sensitive to certain contaminants than analogous species and ecosystems in temperate and tropical areas [7, 8]. Unique characteristics, such as higher-lipid content, a tendency to brood young, longer life spans, gigantism, long developmental stages and slow metabolic rates may all elevate the sensitivity of high latitude marine taxa to contamination [7]. Previous studies have found high latitude species to be particularly sensitive to copper when compared to related species of the same life stage in lower latitudes [9-12]. As processes in polar regions are slower, recovery times from a contamination event are longer, while heightened sensitivities of abundant invertebrate species to contaminants could cause longer lasting bottom-up trophic cascading effects. [7]. The characteristics of high latitude biota clearly indicate that environmental guidelines developed in temperate areas cannot be used in high latitude regions, and highlight the requirement for regional specific guidelines.

The development of water quality guidelines using data from toxicity tests involves several provisions to ensure adequate protection for local biota. Each species should be tested at its most sensitive life stage, in order to provide protection across its entire life span [13]. In addition, environmental guidelines for specific regions need to be developed based on local native flora and fauna, and should include a minimum of 5-10 species, with over 15 species being the optimum number [14, 15]. These taxa should include ecologically important and relatively sensitive species [13, 16].

It is widely accepted that early life stages are more sensitive than adults to many environmental stressors, including contaminants [13]. However, a number of studies that compare life stages reveal that the most sensitive stage in a species' life history can differ between species and is dependent on the contaminant in question [17, 18]. A review by Hutchinson et al. [19], challenged the notion of a general decrease in sensitivity with age; they demonstrated through comparisons of EC50 estimates that juvenile aquatic invertebrates were more sensitive than adults for just 1 of the 12 chemicals tested. While there are few studies comparing the sensitivity of different life stages to environmental stressors and contaminants in polar regions, it was revealed that juveniles of Antarctic marine invertebrates are more resistant to warming and hypoxia than adults [20, 21].

The slow development of high latitude species highlights the need for comparative toxicity data between life stages, as slower development at early life stages could mean that high latitude species spend longer at more vulnerable stages [9]. Longer test durations should also be used in toxicity tests with these species in order to account for differences in their physiology and to accurately determine their sensitivities for guideline development [9, 11]. The identification of higher sensitivity at a particular stage may reduce the need for lengthy test durations with high latitude species, saving time and resources.

Copper is a major contaminant in polar coastal zones, being common in wastewater discharges [22], found in legacy waste sites and fuel spills associated with polar research stations [1, 3], and with increasing use on ship hulls since the banning of TBT [23]. Concentrations of copper may therefore be increasing in subantarctic areas with the intensifying shipping activities associated with research, fishing and tourism. Copper has been found to be one of the most toxic metals to aquatic biota in comparison to other metal contaminants in tropical and temperate regions [7, 24, 25] .

The aim of this study was to compare the sensitivity of different life stages of several subantarctic marine invertebrates to copper. We selected four species from different phyla: the isopod *Limnoria stephenseni* (Menzies 1957), the bivalve *Gaimardia trapesina* (Lamarck 1918), the gastropod *Laevilittorina caliginosa* (Gould 1849) and the flatworm *Obrimoposthia ohlini* (Bergendal 1899). In addition to mortality, we aimed to determine appropriate sublethal measures as alternative endpoints for each species. Lastly, we aimed to determine which of these species is most appropriate for future culturing and use in toxicity assessments for subantarctic regions. Individuals used in toxicity tests were collected from Macquarie Island. This island is representative of many other subantarctic islands, as well as areas of the Antarctic Peninsula and southern South America with similar climates. Findings from this study should therefore be applicable to the development of water quality guidelines and risk assessments procedures across the whole subantarctic region.

METHODS

Study location and species

The four species used in this study were collected from subantarctic Macquarie Island (54.6167° S, 158.8500° E), just north of the Antarctic Convergence in the Southern Ocean. Sea temperatures surrounding Macquarie Island are relatively stable throughout the year, with average temperatures ranging from ~4 to 7 °C [26]. Collection sites were free from any obvious signs of contamination and did not have elevated concentrations of metals as confirmed by analysis of seawater samples taken from the collection sites by inductively coupled plasma optical emission spectrometry (ICP-OES; Varian 720-ES)(Supplemental data).

Toxicity tests were conducted at Macquarie Island over the 2013/14 austral summer, and at the Australian Antarctic Division (AAD) in Tasmania, Australia, from 2013 to 2015 (Table 1). The aquarium within the Marine Research Facility at the AAD used for culturing and for holding biota prior to their use in tests was maintained at a temperature of 5.8°C under recirculating conditions (at 0.49 L/sec). Test specimens that were used in tests on Macquarie Island, rather than at the AAD, were acclimated to laboratory conditions 24 to 48 h prior to commencement of tests (Table 1).

Each species inhabited different areas within the intertidal and subtidal zones and all were highly abundant in each of their respective habitats. The gastropod *Laevilittorina caliginosa* was collected from pools high on the intertidal zone; the flatworm *Obrimoposthia ohlini* from the undersides of boulders from the intertidal to shallow subtidal areas; the bivalve *Gaimardia trapesina* from several macroalgae species in high energy locations in the shallow subtidal; and the isopod *Limnoria stephenseni* from the floating fronds of the kelp *Macrocystis pyrifera*, which were located several hundred meters offshore.

Adults of each species collected from the field were within a narrow size range to minimise differences in age between individuals tested which were unknown (Table 1). The smaller size class of bivalves tested (juveniles: 2.5 ± 0.5 mm, Table 1) was collected from the field along with the adults (8.0 ± 1.0 mm, Table 1). Based on knowledge of the growth rate of this species (0.8 mm per year; [27]), the smaller size class likely represents a young adult of approximately

2.5 to 4 y old, as opposed to a juvenile stage, and adults collected were approximately 9 to 11 y old.

Juvenile flatworms, isopods and gastropods were all sourced as the result of reproduction in the laboratory at the AAD, and hence their approximate age at testing was known. The flatworms hatched from small (2 mm diameter) brown eggs, laid on rocks or on the sides of aquaria. The flatworms exhibited age based morphological differences; juvenile flatworms were light grey in colour, while the adults were black. The gastropods hatched from small (1 mm diameter) translucent eggs laid on weed, often in a cluster. For flatworms and gastropods, differing hatching times between mature female individuals resulted in an age range of 2 weeks to 3 months. In contrast, juvenile isopods were all the same age. Although brooding isopods were not observed, juveniles were noticed during routine feeding, thus were likely within 2-3 days of being released, 6 months after adults were brought from the field to the aquarium. The tests with these juvenile isopods were done within 1 week of being observed within aquaria.

Toxicity tests

A static non-renewal test regime was used for all toxicity tests. Two replicate tests were conducted for each species at each life stage, with the exception of the juvenile isopods, where due to the limited number of individuals available, only one test was completed. Longer tests durations of 14 days were used for acute responses due to the longer life span and slower response of the subantarctic species to contaminants compared to temperate and tropical species as determined in previous studies [11, 12].

All experimental vials and glassware were washed in 10% nitric acid and rinsed thoroughly with MilliQ water three times before use. Tests were done in lidded polyethylene vials of varying sizes, depending on the size and number of individuals used in tests (Table 1). Water was not aerated as Dissolved oxygen (DO) levels remained relatively high for the duration of tests due to high dissolution rates in cold water. Acid washed and Milli-Q rinsed mesh (600 µm nylon) was provided for isopods to rest on, while no structure was added to vials for the other test species. Test solutions were prepared 24 h prior to the addition of invertebrates. Five copper concentrations in seawater were prepared using a 500 mg/L Univar analytical grade $CuSO_4$ in MilliQ stock solution, plus a control for each test. Seawater was filtered to 0.45 µm, and water quality parameters were measured using a TPS 90-FL multimeter at the start (d 0) and end (d 14) of tests. Dissolved oxygen was >80% saturation, salinity was 33 to 35 ppt, and pH was 8.1 to 8.3 at the start of tests. Tests were kept in controlled temperature cabinets set at 6°C under 16:8h light:dark during the summer, and 12:12h for tests during the rest of the year (light intensity of 2360 lux). Temperatures within cabinets were monitored throughout tests using Thermochron iButton data loggers.

Samples from one replicate from each treatment test solution were taken at the start (day 0) and end (day 14) of tests. Samples were filtered through an acid washed and Milli-Q rinsed, 0.45µm Minisart syringe filter and acidified with 1% ultra-pure nitric acid before being analysed by ICP-OES to determine dissolved metal concentrations (QA/QC in supplemental data). Measured copper concentrations at the start of tests were within 96% of nominal target concentrations. Measured concentrations at the start and end of tests were averaged to estimate exposure concentrations, which were subsequently used in statistical analyses to determine point estimates (Supplemental data). Both survival and sublethal (behavioral) endpoints were assessed to determine sensitivity to copper. Vials were checked daily and observations recorded of individual responses on days 1, 2, 4, 7, 10 and 14. Tests were terminated when surviving individuals occurred in less than two concentrations, which was generally at 14 d for all species except for bivalves (7 to 10 d). Gastropods were scored as dead when their operculum was open and there was no response to stimulus (touch of a probe) on the operculum. Flatworms were scored as dead when there was no movement. Bivalves were scored as dead when there was no movement and when the shells were gaping open due to dysfunctional adductor muscles. Isopods were scored as dead when there was no movement of any appendages.

The behavioral endpoint scored for each species was attachment, which indicated healthy and active individuals. For gastropods, this meant the foot was fully extended and attached to experimental vials; for flatworms, the whole body was able to attach (as those affected by copper appeared slightly contracted and could not lie flat); for bivalves, the byssal threads were used to fix individuals to the bottom of the vial, with the siphon also visible and shell slightly open for water exchange; and for isopods, individuals were either holding onto the mesh or were swimming, in which case they often reattached to the mesh during observation.

Data analysis

LC50 (concentrations that resulted in 50% mortality in the test population) and EC50 (concentrations that resulted in 50% of the test population becoming unattached) were determined for each observation time. Either Maximum Likelihood Probit, Trimmed Spearman Karber models or Non-linear Interpolation were used to determine each estimate (depending on conforming with model assumptions) using the software ToxCalc (version 5.0, Tidepool Scientific Software). Estimates for 1 and 2 d for all species are not shown, due to the limited responses observed.

RESULTS

Juveniles of three of the four species tested were generally more sensitive than the adults (Figures 1-4). Juveniles of the isopod *Limnoria stephenseni* were more sensitive than adults at 4 d, but after 4 d, sensitivity did not differ between life stages (Figure 1). Juveniles of the flatworm *Obrimoposthia ohlini* were consistently more sensitive than adults, for mortality (LC50) and attachment behavior (EC50) with the exception of attachment at day 4, where there was little difference (Figure 2). Juvenile gastropods, *Laevilittorina caliginosa*, were more sensitive than adults, which was particularly apparent at 4 and 7 d for attachment (Table 2; Figure 3). Mortality of gastropods was low at most of the concentrations tested for the duration of the tests, thus LC50 values could generally not be determined (Table 3). In contrast to these three species, adult bivalves *Gaimardia trapesina* appeared to be more sensitive than young adults at all days tested and for both lethal and sublethal endpoints (Figure 4). The EC10 and LC10 values (Supplemental data) showed similar patterns to the EC50 and LC50 values (Table 2; Table 3).

Sensitivity to copper varied between species. The bivalve was the most sensitive by a large margin for both life stages, as shown by both EC50 and LC50 estimates and which could not be calculated in some instances between 7-14 d due to high mortality across concentrations (Table 2; Table 3). The gastropod was the least sensitive, with high survival at most concentrations tested in comparison to the other species (Table 3). The flatworm and isopod were of intermediate sensitivity, as shown by both their EC50 and LC50 estimates (Table 2; Table 3).

Behavior was affected at lower copper concentrations than was survival for the bivalve, gastropod and flatworm, but not for the isopod (Figures 1-4). For the isopod, the difference in sensitivity between behavior and survival was very small, as indicated by very similar EC50 and

LC50 estimates (Figure 1; Tables 2-3). For adult flatworms, the difference between EC50 and LC50 values was notably large at the beginning of the test, while by 14 d EC50 and LC50 estimates were similar (Figure 2). While gastropods could survive exposure to high concentrations of copper, their ability to remain attached was compromised at lower concentrations than either the flatworm or isopod, across all days and for both life stages (Figure 3; Table 2).

Control survival was generally 100% in all tests with all species up to 10 d. In some tests, this decreased to 80 to 95% by day 14. Water quality generally remained stable and within acceptable limits throughout most tests up to 14 d (temperature= 6 ± 1 °C, salinity= 33-35 ppt, DO= 70-100% saturation, pH= 7.6 -8.2). Although control survival was not affected (96–100% throughout the tests), the greatest water quality decline was observed in tests with both adult and juvenile bivalves, with pH dropping to between 6.8 and 7.2 by 14 d. This decreased pH may have increased the observed metal toxicity, therefore results should be interpreted with caution.

DISCUSSION

The sensitivity of juveniles to copper was higher than that of adults for the gastropod, flatworm and isopod. These results are in agreement with previous findings that early life stages of aquatic biota are often more sensitive to contaminants than adults. Heightened sensitivity to contaminants at earlier life stages has been reported for freshwater gastropods [28, 29], bivalves [18], water fleas [17], and fish [19], as well as marine species such as coral [30] copepods [31, 32] amphipods [18, 33] and mysid [34]. Comparisons of any life stage of polar biota are rare. Sensitivity to copper increased through the larval development of the Antarctic sea urchin *Sterechinus neumayeri* [9], and juvenile Antarctic amphipods *Paramorea walkeri* were more sensitive to copper than adults [35]. Previous researchers have suggested that physiological mechanisms underpin differences in sensitivity between life stages, including a higher surface to volume ratio and reduced energy reserves for maintenance at earlier life stages [17, 19]. Our findings provide further evidence that the patterns observed in previous studies are consistent with subantarctic marine invertebrates; with early life stages generally more sensitive than adults.

The bivalve was the only test species in this study to deviate from the general pattern of higher sensitivity of earlier life stages. This result suggests that there are exceptions to the general trend of higher sensitivities in earlier life stages, as suggested by other studies [17, 19]. While no previous studies have compared the sensitivity of life stages of Antarctic bivalves to contaminants, one study has shown that juvenile *Laternula elliptica*, were more tolerant to increased temperature than were adults [20]. The results in the present study are somewhat inconclusive due to confounding factors within the test, which may have influenced results. For example, pH decreased considerably in tests with older/larger adults (6.8) and with younger/smaller adults (7.1) from the initial pH of ~8.1. With possible interactive effects of a lowered pH with copper, toxicity is likely to have been enhanced, especially for the older/larger adults. Ammonia may also have influenced test results, but was not measured in this study. Despite these possible additional stressors due to changes in water quality, survival of bivalves in controls was not impacted and was consistently >96% throughout the tests. Investigations on pH tolerance, ammonia, and other water quality parameters may be beneficial for refinement of tests with this species in the future. In addition, toxicity testing incorporating water renewals in order to maintain water quality throughout tests may further inform comparative toxicity between bivalve life stages and determine whether older/larger adult stages of this species really are more sensitive than are younger/smaller adults.

This study highlights the importance of testing a range of species when developing water quality guidelines, due to the high variation in species sensitivities. The gastropod *L. caliginosa* is likely well adapted to environmental stressors and a variable environment, due to its position high on the intertidal zone [36]. It is likely able to use adaptations, such as operculum closure, to tolerate unfavourable conditions such as copper contamination. In contrast, both life stages of the bivalve *G. trapesina* were highly sensitive to copper. With their position low in the intertidal zone, they likely possess fewer adaptations for surviving adverse and variable environmental conditions and extremes. This correlation between contaminant sensitivity and position on the shore has been observed previously for molluscs [12, 37] and for a tropical copepod [38].

Water quality guidelines do not currently exist for subantarctic regions, and it is apparent that guidelines developed in temperate and tropical regions may not be appropriate for polar regions due to differences in species sensitivities [7]. While the species in this study appear not to be particularly sensitive, greater sensitivities to contaminants have been observed in other high latitude species including copepods [10, 11], gastropods [12], algae [35] and urchins [9], when compared to related species in lower latitudes. For example, two other adult subantarctic gastropods, *Macquariella hamiltoni* (7 d LC50 of 78 µg/L) and *Cantharidus capillaceus coruscans* (7 d LC50 of 29 µg/L) were highly sensitive when compared to adult low latitude species [12]. Testing of embryonic and larval stages, as commonly done for temperate and tropic mollusc test species, would likely reveal even higher sensitivities. Nevertheless, the present study contributes valuable additional sensitivity information, including estimates for early life stages that can be incorporated into the development of region specific water quality guidelines.

Further, this study corroborates previous studies that highlight the need for specific conditions in toxicity tests with biota from higher latitudes [9, 10, 39]. For all species, no significance response was observed up to 4 d exposure, with a steep decline in survival occurring between 4 and 7 d for both the flatworm and isopod. This is in contrast to tests with related temperate and tropical species, where responses are generally observed within 1 to 4 d, and test durations for acute tests are rarely greater than 4 d. This slower response of subantarctic biota to metals indicates that uptake and processing of contaminants by subantarctic species at lower temperatures occurs at slower rates than in species from other regions. This aligns with previous work with Antarctic species, where toxicity tests can be up to 14 to 30 d long in order to illicit a response [10, 40].

Sublethal endpoints were useful early indicators of copper toxicity in this study and confirmed patterns of toxicity based on survival that were observed between life stages. Sublethal endpoints also allowed for comparisons in sensitivity between life stages for the gastropod, where the determination of mortality proved difficult, due to operculum closure. In all cases, sublethal effects were precursors to lethal effects that occurred over longer exposure periods and, importantly, were detected at lower concentrations. This was particularly apparent for adult flatworms, for which EC50 estimates were much lower than LC50 estimates at 4 and 7 d, but by 14 d EC50 and LC50 estimates were similar (Figure 2). This suggests that short term (4 d) EC50 estimates based on behavior give an early indication of likely LC50 estimates at 14 d, as individuals with behavior affected at 4 d will have died by 14 d. Sublethal effects are being increasingly used in acute toxicity studies [41, 42], to determine effects at lower concentrations and over shorter exposure periods, as more evidence accumulates of the link between sublethal effects and subsequent mortality [43]. This is particularly useful for tests with cold-water species, which generally necessitate longer exposure periods [9, 10, 39]. The development of sublethal endpoints can potentially reduce the need for impractical and costly long test durations. Other sublethal endpoints that could be considered for future research with these species include reproduction and larval development, particularly for *L. caliginosa* and *O. ohlini*, where embryonic development could be observed after eggs had been laid. As many other subantarctic species brood their young, including *G. trapesina* and *L. stephenseni*, testing of embryonic development may be difficult as it would require simulating a brooding environment, rather than a broadcasting environment.

In the potential absence of an ability to test a larger range of subantarctic species for further toxicity testing and guideline development, we recommend the flatworm *O. ohlini* and the isopod *L. stephenseni* as suitable test species. While they were not as sensitive as the bivalve *G. trapesina*, they were both abundant in the field, were easily collected, can be maintained in aquaria, were amenable to testing, and both sublethal and lethal endpoints were clear and less likely to be subjective in assessments. This is in contrast to the gastropod *L. caliginosa* where closure of the operculum created difficulties when determining mortality. The flatworm was also easy to culture and many juveniles were released in the aquaria during the course of this study. Culturing of the isopod may also be possible, but further work is required to better understand the reproductive biology of this species.

This study highlights the need for specific water quality guidelines for Polar Regions. To do so requires determination of the sensitivity of key biota. This study therefore contributes significantly towards the development of such regional specific guidelines. We conclude that, as with biota in other regions, early stages of most of these species should be targeted for use in toxicity tests. Further testing with larval stages and comparisons between life stages of other local biota will complement these findings of heightened sensitivity of both high latitude species and earlier life stages.

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Data availability

A metadata record for this work is publically available (Holan J, King C, Davis A. 2017. Comparative copper sensitivity between life stages of common subantarctic marine invertebrates. Australian Antarctic Data Centre. doi:10.4225/15/5934e597b1c8e)

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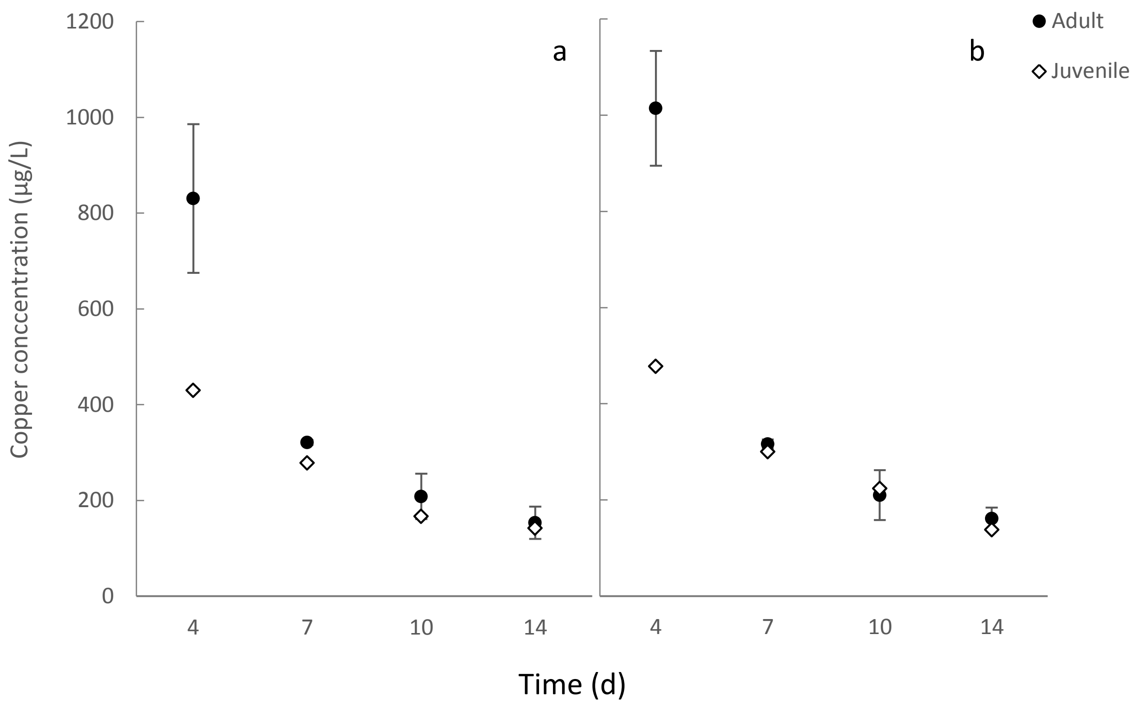

Figure 1. Average EC50 (a) and LC50 (b) values for copper for adults (black circle) and juveniles (white diamond) of the isopod *Limnoria stephenseni*. EC50 estimates are based on "attachment" behavior which was indicative of good health. Averages are based on 2 tests for adults while only one test was done for juveniles. Error bars represent 1 standard error.

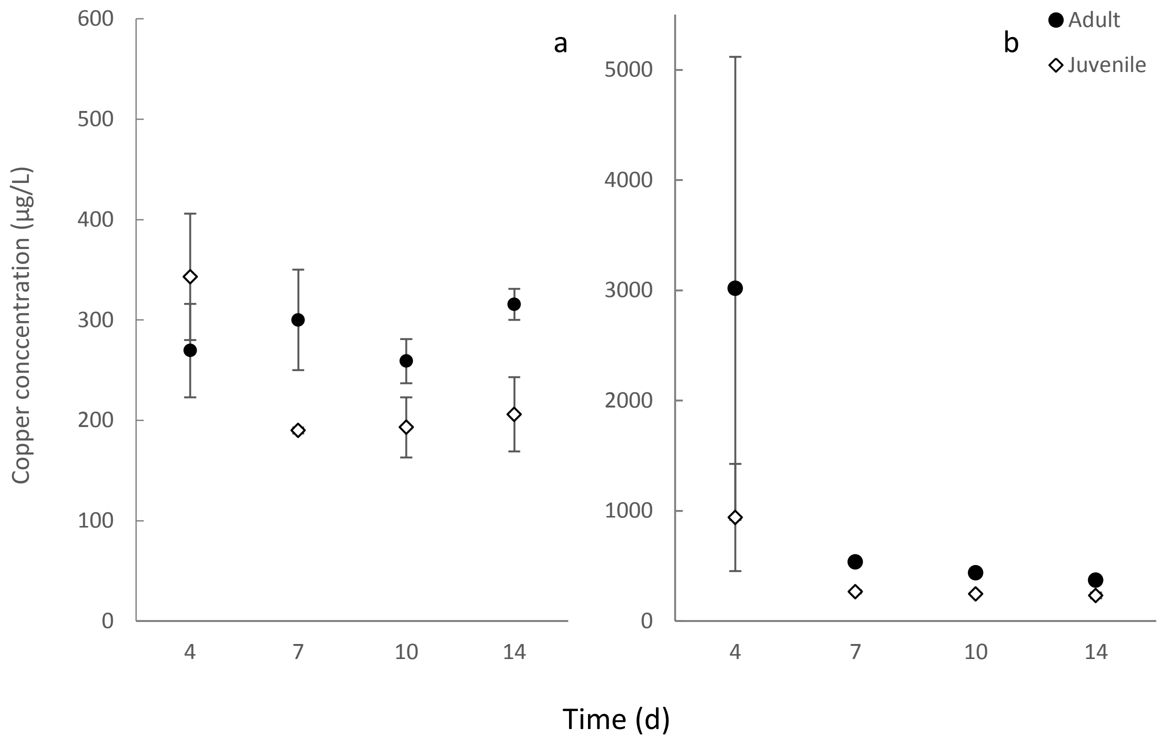

Figure 2. Average EC50 (a) and LC50 (b) values for flatworm *Obrimoposthia ohlini*. Same format as Figure 1. Averages are based on 2 tests.

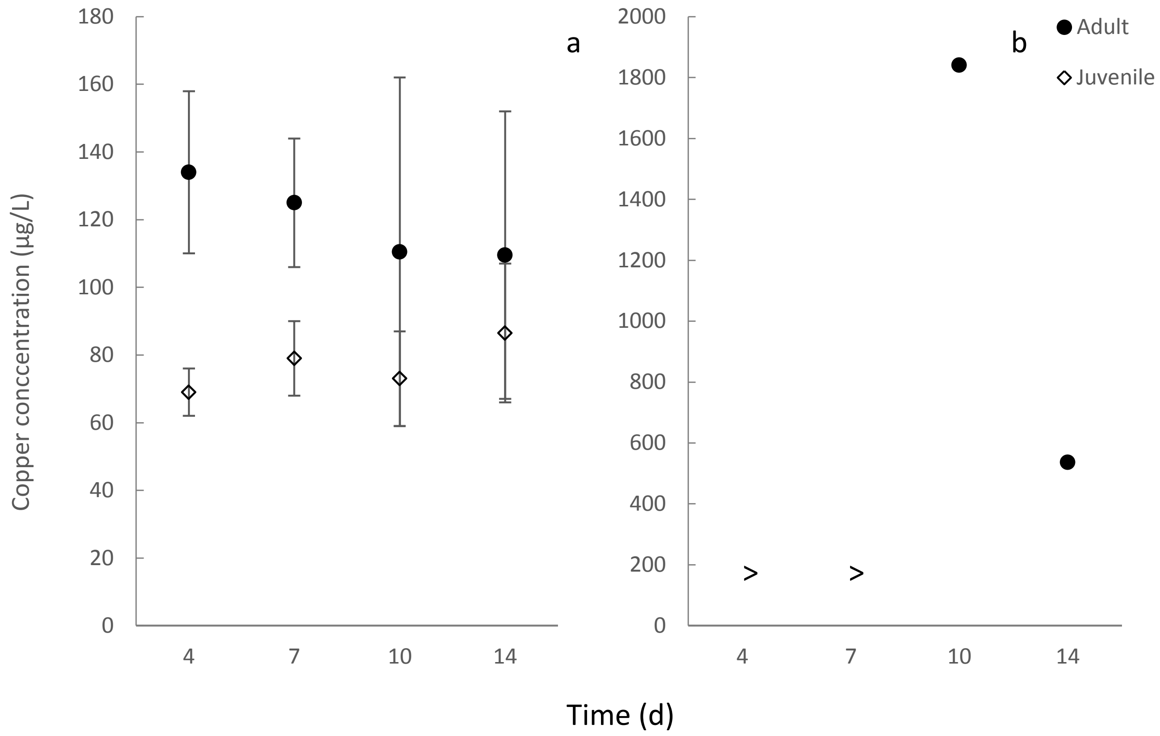

Figure 3. Average EC50 (a) and LC50 (b) values for the gastropod *Laevilittorina caliginosa.* Same format as Figure 1. Averages are based on 2 tests. A “>” indicates data not shown due to low mortality at concentrations tested (Table 4).

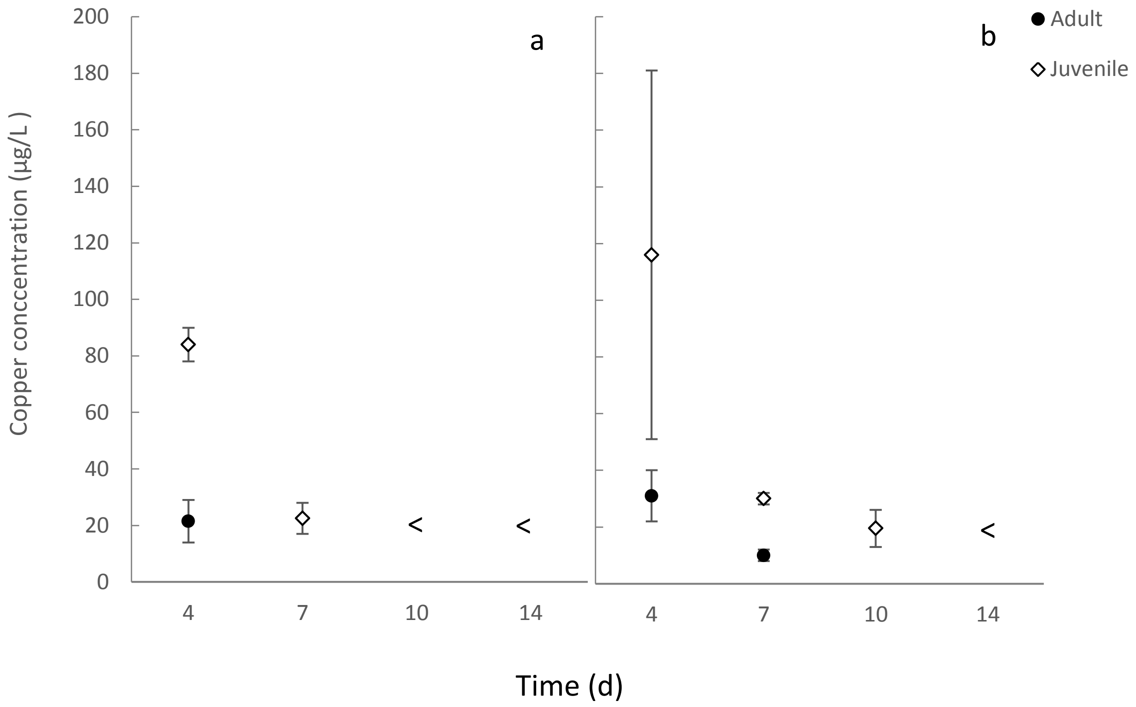

Figure 4. Average EC50 (a) and LC50 (b) values for the bivalve *Gaimardia trapesina*. Same format as Figure 1. Averages are based on 2 tests. A"<"indicates data not displayed due to high mortality (Table 4). These results should be interpreted with caution due to compromised water quality during tests.

Table 1. Toxicity test conditions for each test species and life stage of subantarctic marine invertebrates. Two replicate tests were done for each species and life stage, with the exception of juvenile *Limnoria stephenseni*.

Species	Vial size (mL)	Water volume (mL)	No. of replicate vials per conc.	No. of individuals per vial	Mean size of individuals ±SD (mm)[a]	Test laboratory location[b]
Adults						
Isopod *Limnoria stephenseni*	200	180	5	10	5.0 ± 1.0	MI
Flatworm *Obrimoposthia ohlini*	200	180	5	10	5.8 ± 1.1	AAD
Gastropod *Laevilittorina caliginosa*	120	100	5	10	3.6 ± 0.6	MI (T1) AAD (T2)
Bivalve *Gaimardia trapesina*	200	180	5	10	8.0 ± 1.0	MI
Juveniles						
Isopod *Limnoria stephenseni*	70	50	3	7	2.0 ± 0.0	AAD
Flatworm *Obrimoposthia ohlini*	70	50	5	10	2.6 ± 0.8	AAD
Gastropod *Laevilittorina caliginosa*	70	50	3-4	6-10	0.8 ± 0.2	AAD
Bivalve[c] *Gaimardia trapesina*	120	100	5	10	2.5 ± 0.5	MI

[a] Measurement of longest dimension, n= 30-40

[b] "MI" refer to the laboratory on Macquarie Island, "AAD" to the Australian Antarctic Division laboratory in Tasmania, Australia, and "T1" and "T2" refers to test numbers 1 and 2.

[c] "Juvenile" bivalves were more likely young adults based on growth rates of 0.8 mm per year, reported in [26]

Table 2. Copper EC50 values (concentrations causing 50% of test individuals to be affected in terms of ability to attach) and 95% fiducial limits for adults and juveniles of four species of subantarctic marine invertebrates over the 14 d test duration.

Cu EC50 (±95% FL) (µg/L)					
	Test #	4 d	7 d	10 d	14 d
Isopod *Limnoria stephenseni*					
	1	675 (518-1035)	323 (279-374)	161 (132-187)	120 (83-143)
	2	986 (634-3776)	316 (280-355)	256 (185-291)	187 (147-220)
	1	430 (286-863)	278 (155-402)	167 (76-223)	142 (63-180)
Flatworm *Obrimoposthia ohlini*					
Adults	1	316 (288-342)	350 (318-381)	327 (297-356)	331 (307-356)
	2	223 (147-259)	250 (202-278)	281 (254-304)	300 (268-327)
Juveniles	1	280 (205-345)	187 (153-226)	163 (136-197)	169 (140-204)
	2	406 (262-1185)	193 (151-256)	223 (185-270)	243 (185-296)
Gastropod *Laevilittorina caliginosa*					
Adults	1	158 (129-189)[b]	144 (137-152)[b]	162 (148-178)[b]	152 (147-157)[b]
	2	110 (67-136)[b]	106 (59-130)[b]	59 (9-97)[b]	67 (3-104)[b]
Juveniles	1	62 (44-78)	68 (45-86)	59 (48-69)	66 (60-73)
	2	76 (25-103)	90 (40-117)	87 (57-101)	107 (101-113)
Bivalve *Gaimardia trapesina*					
Adults	1	14 (11-16)	< 10[a]	< 10 [a]	< 10[a]
	2	29 (25-32)	< 21[a]	< 21[a]	< 21[a]
Juveniles	1	129 (112-147)	17 (2-30)	< 43[a]	< 43[a]
	2	39 (24-54)	28 (18-37)	< 13[a]	< 13[a]

[a]A "less than" symbol (<) indicates significant mortality in the lowest concentration, thus an estimate could not be determined and the lowest test concentration is given.
[b] Reported in [12]

Table 3. Copper LC50 values (concentrations causing 50% mortality of test individuals) and 95% fiducial limits for adults and juveniles of four species of subantarctic marine invertebrates over the 14 d test duration.

Cu LC50 (±95% FL) (µg/L)					
	Test #	4 d	7 d	10 d	14 d
Isopod *Limnoria stephenseni*					
Adults	1	895 (649-1644)[d]	313 (265-325)	158 (138-180)	138 (128-149)
	2	1134 (681-6647)[d]	326 (288-368)	262 (184-297)	184 (145-217)
Juveniles	1	478 (333-1456)	300 (197-474)	224 (143-292)	138 (76-180)
Flatworm *Obrimoposthia ohlini*					
Adults	1	> 623[a]	560 (511-635)[b]	450 (410-495)[b]	401 (366-436)
	2	917 (847-997)	514 (389-650)[b]	425 (388-461)[b]	342 (313-370)
Juveniles	1	455 (377-555)	251 (204-296)	237 (193-281)	206 (169-246)
	2	> 438[a]	282 (216-423)	258 (198-318)	258 (198-318)
Gastropod *Laevilittorina caliginosa*					
Adults	1	> 686[a]	> 686[a]	1841(1044-31986)[de]	537 (426-741)[de]
	2	> 1487[a]	> 1487[a]	> 1487[a]	> 1487[a]
Juveniles	1	> 629[a]	> 629[a]	> 629[a]	> 629[a]
	2	> 364[a]	> 364[a]	> 364[a]	> 364[a]
Bivalve *Gaimardia trapesina*					
Adults	1	22 (4 - 43)	12 (10-13)	< 10[c]	< 10[c]
	2	40 (13 -88)	< 21[c]	< 21[c]	< 21[c]
Juveniles	1	354 (211-1237)	32 (8-52)[b]	13 (0-27) [b]	< 43[c]
	2	51 (36-72)	28 (25-31)[b]	26 (21-29)[b]	< 13[c]

[a]A “greater than” symbol (>) indicates the highest concentration with no response, or that the estimate was unable to be calculated.
[b]Reported in [11]
[c]A “less than” symbol (<) indicates significant mortality in the lowest concentration, thus an estimate could not be determined and the lowest test concentration is given
[d]Estimate is outside the range of concentrations tested
[e]Reported in [12]

S1. Copper concentrations (µg/L)[a] used in each test, determined by the average of the measured concentration at the start (d 0) and end (d 14) of tests for each treatment

	Adult	Juvenile
Isopod		
Limnoria stephenseni		
Test 1	0, 84, 178, 366, 534, 725	0, 30, 77, 168, 260, 519
Test 2	0, 82, 166, 333, 534	[b]NT
Flatworm		
Obrimoposthia ohlini		
Test 1	0, 38, 82, 254, 418, 609	0, 43, 93, 243, 459, 664
Test 2	0, 278, 463, 634, 984, 1332	0, 50, 105, 212, 439
Gastropod		
Laevilittorina caliginosa		
Test 1	0, 69, 101, 220, 476, 686	0, 38, 79, 164, 351, 629
Test 2	0, 133, 280, 647, 992, 1488	0, 89, 138, 190, 279, 364
Bivalve		
Gaimardia trapesina		
Test 1	0, 10, 27, 57, 115, 173	0, 43, 85, 162, 267, 610
Test 2	0, 21, 27, 47, 60, 80	0, 13, 29, 41, 62, 75

[a]Measured using inductively coupled plasma optical emission spectrometry (ICP-OES)
[b]NT indicates no test was done (due to only one spawning event)

S2. Copper EC10 values and 95% fiducial limits for adults and juveniles of four species of subantarctic marine invertebrates. Effect concentrations are based on the individual's ability to attach

Cu EC10 (±95% FL) (µg/L)					
		Exposure duration			
	Test #	4 d	7 d	10 d	14 d
Isopod *Limnoria stephenseni*					
Adults	1	144 (55-219)	138 (83-185)	84 (57-107)	79 (37-103)
	2	262 (157-349)	167(81-228)	189 (96-233)	124 (73-155)
Juveniles	1	186 (13-282)	110 (12-182)	82 (11-132)	82 (11-122)
Bivalve *Gaimardia trapesina*					
Adults	1	6 (4-8)	< 10[a]	< 10 [a]	< 10[a]
	2	13 (9-16)	< 21 [a]	< 21 [a]	< 21 [a]
Juveniles	1	56 (43-68)	5 (0-14)	< 43 [a]	< 43 [a]
	2	31 (25-35)	22 (16-25)	15 (10-18)	< 13 [a]
Flatworm *Obrimoposthia ohlini*					
Adults	1	214 (176-241)	224 (182-255)	216 (175-246)	248 (215-272)
	2	143 (57-190)	172 (101-210)	207 (158-235)	211 (166-242)
Juveniles	1	125 (60-178)	99 (70-124)	96 (70-117)	105 (75-129)
	2	58 (15-95)	56 (29-80)	120(57-164)	118 (45-172)
Gastropod *Laevilittorina caliginosa*					
Adults	1	90 (62-114)	131 (106-144)	110 (31-131)	109 (27-113)
	2	54 (16-81)	56 (58-129)	18 (1-45)	26 (0-60)
Juveniles	1	29 (14-41)	34 (14-50)	38 (24-47)	44 (27-48)
	2	37 (3-63)	49 (8-76)	61 (21-78)	29 (0-156)

[a]A "less than" symbol (<) indicates significant mortality in the lowest concentration, thus an estimate could not be determined and the lowest test concentration is given.

S3. Copper LC10 values and 95% fiducial limits for adults and juveniles of four species of subantarctic marine invertebrates

Cu LC10 (±95% FL) (µg/L)					
		Exposure duration			
	Test #	4 d	7 d	10 d	14 d
Isopod *Limnoria stephenseni*					
Adults	1	153 (81-213)	118 (85-147)	82 (64-98)	NC[a]
	2	279(165-384)	167 (88-341)	192 (90-238)	122 (72-152)
Juveniles	1	173 (14-267)	98 (15-62)	104 (28-156)	71 (17-108)
Bivalve *Gaimardia trapesina*					
Adults	1	8 (6-14)	6 (5-8)	< 10[b]	< 10[b]
	2	22 (16-27)	17 (12-22)	< 21 [b]	< 21 [b]
Juveniles	1	80 (22-127)	14 (12-16)	4 (0-15)	< 43 [b]
	2	30 (8-40)	22 (16-25)	20 (12-24)	< 13 [b]
Flatworm *Obrimoposthia ohlini*					
Adults	1	>623[c]	350 (285-394)	281 (224-322)	263 (215-298)
	2	574 (494-638)	328 (155-421)	267 (222-304)	255 (216-283)
Juveniles	1	168 (96-226)	144(96-181)	134 (91-169)	115 (80-144)
	2	NC[a]	72 (35-104)	132 (56-181)	132 (56-181)
Gastropod *Laevilittorina caliginosa*					
Adults	1	NC[a]	NC[a]	546 (383-865)	129 (86-170)
	2	NC[a]	NC[a]	NC[a]	NC[a]
Juveniles	1	NC[a]	415 (160-1747)	229 (120-186)	NC[a]
	2	NC[a]	NC[a]	284 (128-403)	234 (123-313)

[a] "NC" indicates estimate could not be calculated due to inability to conform to the models
[b] A "less than" symbol (<) indicates significant mortality in the lowest concentration, thus an estimate could not be determined and the lowest test concentration is given.
[c] A "greater than" symbol (>) indicates the highest concentration with no response, or that the estimate could not be calculated

S4. Inductively coupled plasma optical emission spectrometry (ICP-OES) quality assurance/quality control (QAQC) details for all ICP-OES results.

Method Detection Limits (MDL's):

Cu (wavelength 213.598) = 2.74 ppb

In-house multi-element standards were made from primary standards (ACR- Cat No. 4367) and matrix matched (uncontaminated filtered seawater). These working solutions were confirmed using matrix matched single element (Cu) standards (ACR). Standard blanks (uncontaminated filtered seawater) were confirmed using Cass-4 SW (Nearshore Seawater Reference Material for Trace Metals) whilst in-house single and multi-element standards were further confirmed with Water QC Standard (Inorganic Ventures - Cat. No. QCP-MTL). Yttrium was used as an internal standard to detect and correct for instrument drift. Duplicates, blanks, CRMs and single element standards were sampled every 15-20 samples and maintained between 90 – 110% of required concentrations. Spike recovery for matrix matched Cu were also 90 – 100% of expected values